JAPAN PATENT OFFICE

This is to certify that the annexed is a true copy of the following application as filed with this office.

Date of Application : December 3, 2003

Application Number : Patent Application No. 2003-404472
[JP2003-404472]

Applicant(s) : RIKEN
MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.

January 13, 2005

Hiroshi OGAWA

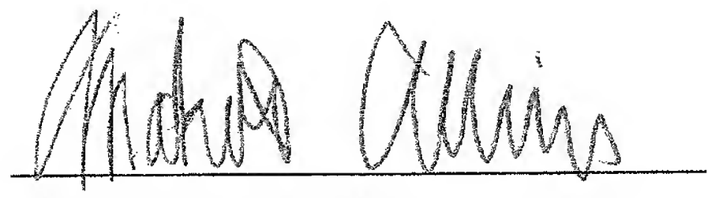
Commissioner, Patent Office

Certification No. 2004-312221

VERIFICATION OF TRANSLATION

I, Makoto AIHARA, Patent Attorney, of SIKs & Co., 8th Floor, Kyobashi-Nisshoku Bldg., 8-7, Kyobashi 1-chome, Chuo-ku, Tokyo 104-0031 JAPAN hereby declare that I am the translator of the certified official copy of the documents in respect of an application for a patent filed in Japan on December 3, 2003 under Patent Application No. 404472/2003 and that the following is a true and correct translation to the best of my knowledge and belief.

Dated: September 17, 2010



Makoto AIHARA

[Title] Application for Patent

[Reference Number] A31701A

[Filing Date] December 3, 2003

[Address] To the Commissioner of the Patent Office

[Inventor]
[Domicile or Residence] c/o RIKEN, 2-1, Hirosawa, Wako-shi, Saitama
[Name] Atsushi MIYAWAKI

[Inventor]
[Domicile or Residence] c/o MEDICAL & BIOLOGICAL LABORATORIES CO., LTD., INALAB., 1063-103, Aza Ohara, Oaza Terasawaoka, Ina-shi, Nagano
[Name] Satoshi KARASAWA

[Inventor]
[Domicile or Residence] c/o MEDICAL & BIOLOGICAL LABORATORIES CO., LTD., INALAB., 1063-103, Aza Ohara, Oaza Terasawaoka, Ina-shi, Nagano
[Name] Toshio ARAKI

[Applicant for Patent]
[Identification Number] 503359821
[Name] RIKEN

[Applicant for Patent]
[Identification Number] 390004097
[Name] MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.

[Administrator]
[Identification Number] 110000109
[Name] SIKs & Co.
[Representative] Masazumi IMAMURA

[Fee]
[Number of register of payment] 170347
[Amount] 21,000

[List of Attached Documents]
[Document's Name] Claims 1
[Document's Name] Specification 1
[Document's Name] Drawings 1
[Document's Name] Abstract 1
[Number of Comprehensive Power of Attorney] 0316216

[Name of Document] CLAIMS

[Claim 1] A fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

[Claim 2] DNA encoding a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

[Claim 3] DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 2; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 2, and which has a nucleotide sequence encoding a protein that has fluorescence properties equivalent to those of the protein encoded by the nucleotide sequence shown in SEQ ID NO: 2 and that exists in the form of a monomer.

[Claim 4] A recombinant vector having the DNA according to claim 2 or 3.

[Claim 5] A transformant having the DNA according to claim 2 or 3 or the recombinant vector according to claim 4.

[Claim 6] A fusion fluorescent protein, which consists of the fluorescent protein according to claim 1 and another protein.

[Claim 7] The fusion protein according to claim 6, wherein another protein is a protein that localizes in a cell.

[Claim 8] The fusion protein according to claim 6 or 7, wherein another protein is a protein specific to a cell organella.

[Claim 9] A method for analyzing the localization or dynamics of a protein in a cell, which is characterized in that the fusion protein according to any one of claims 6 to 8 is allowed to express in the cell.

[Claim 10] A fluorescent reagent kit, which comprises: the fluorescent protein of claim 1; the DNA of claim 2 or 3; the recombinant vector of claim 4; the transformant of claim 5; or the fusion protein of any of claims 6 to 8.

[Name of Document] SPECIFICATION

[Title of Invention] FLUORESCENT PROTEIN

[TECHNICAL FIELD]

[0001]

The present invention relates to a novel fluorescent protein which exists in the form of a monomer. More specifically, the present invention relates to a novel fluorescent protein monomerized by introducing a mutation into a florescent protein derived from Fungia sp., and a use thereof.

[BACKGROUND ART]

[0002]

Green fluorescent protein (GFP) derived from Aequorea victoria, a jellyfish, has many purposes in biological systems. Recently, various GFP mutants have been produced based on the random mutagenesis and semi-rational mutagenesis, wherein a color is changed, a folding property is improved, luminance is enhanced, or pH sensitivity is modified. Fluorescent proteins such as GFP are fused with other proteins by gene recombinant technique, and monitoring of the expression and transportation of the fusion proteins is carried out.

[0003]

One of the most commonly used types of GFP mutant is Yellow fluorescent protein (YFP). Among Aequorea-derived GFP mutants, YFP exhibits the fluorescence with the longest wavelength. The values ε and Φ of the majority of YEPs are 60,000 to 100,000 $M^{-1}cm^{-1}$ and 0.6 to 0.8, respectively (Tsien, R. Y. (1998). Ann. Rev. Biochem. 67, 509-544). These values are comparable to those of the general fluorescent group (fluorescein, rhodamine, etc.). Accordingly, improvement of the absolute luminance of YFP is nearly approaching its limit.

[0004]

In addition, cyan fluorescent protein (CFP) is another example of the GFP mutant. Of this type of protein, ECFP (enhanced cyan fluorescent protein) has been known. Moreover, red fluorescent protein (RFP) has been isolated from sea anemone (Discoma sp.). Of this type of protein, DasRed has been known. Thus, 4 types of fluorescent proteins, that are, green fluorescent protein, yellow fluorescent protein, cyan fluorescent protein, and red fluorescent protein, have successively been developed. The range of the spectrum has significantly been expanded.

[0005]

Previously, the present inventors had succeeded in amplifying a fluorescent protein gene from among the cDNA library of Fungia sp., using preferred primers designed based on the amino acid sequence of a known fluorescent protein, and then cloning it. Thereafter, the present inventors had determined the fluorescence properties of the obtained fluorescent protein derived from Fungia sp. As a result, the present inventors had found that the above fluorescent protein has desired fluorescence properties (International Publication WO03/54191).

[0006]

[Non-patent document 1] Tsien, R. Y. (1998). Ann. Rev. Biochem. 67, 509-544

[Patent document 1] International Publication WO03/54191

[DISCLOSURE OF THE INVENTION]

[Object to be solved by the invention]

[0007]

The molecular weight of the fluorescent protein Kusabira-Orange (KO) isolated from Fungia sp. of Scleractinia, described in International Publication WO03/54191, was measured. As a result, the molecular weight was found to be 70 kDa (the molecular weight calculated from the amino acid sequence thereof was 26 kDa). It is considered that this fluorescent protein usually forms a dimer. In recent years, the demand for labeling cells or molecules with a fluorescent protein has rapidly grown. When cells are

labeled, even if a fluorescent protein forms a multimer, there are no problems because such a fluorescent protein only floats in the cytoplasm. However, when molecules are labeled, such a fluorescent protein that forms a multimer is problematic. For example, when molecules to be labeled form a multimer, there is a possibility that both the target molecule and a fluorescent protein molecule form multimer and that as a result, they form an enormous polymer. In addition, when the formation of a multimer by either one of them is inhibited, such a molecule that cannot form a multimer loses its original properties. Even in a probe of intramolecular FRET (fluorescence resonance energy transfer) wherein multiple fluorescent proteins are used, when fluorescent proteins that form multimer are allowed to express as a single peptide chain, both proteins form multimer, and as a result, the observation of FRET becomes difficult. It is an object of the present invention to solve the aforementioned problems. Specifically, it is an object of the present invention to provide a novel fluorescent protein, which exists in the form of a monomer without forming a multimer.

[0008]

As a result of intensive studies directed towards achieving the aforementioned objects, the present inventors have succeeded in estimating a multimer interface from the amino acid sequence of the protein KO described in International Publication WO03/54191, substituting amino acids on such a multimer interface with other amino acids, and further in monomerizing KO, so that it can maintain fluorescence properties. Moreover, the present inventors have examined the fluorescence properties of the obtained monomer fluorescent protein. As a result, they have found that it has desired fluorescence properties. The present invention has been completed based on these findings.

[0009]

Thus, the present invention provides a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

[0010]

Further another aspect of the present invention provides DNA encoding a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

[0011]

Further another aspect of the present invention provides DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 2; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 2, and which has a nucleotide sequence encoding a protein that has fluorescence properties equivalent to those of the protein encoded by the nucleotide sequence shown in SEQ ID NO: 2 and that exists in the form of a monomer.

[0012]

Further another aspect of the present invention provides a recombinant vector having the DNA according to the present invention as mentioned above.

Further another aspect of the present invention provides a transformant having the DNA or the recombinant vector according to the present invention as mentioned above.

Further another aspect of the present invention provides a fusion protein, which consists of the protein according to the present invention as mentioned above and another protein. Preferably, said another protein is a protein that localizes in a cell. More preferably, said another protein is a protein specific to a cell organella.

[0013]

Further another aspect of the present invention provides a method for analyzing the localization or dynamics of a protein in a cell, which is characterized in that the fusion protein according to the present invention as mentioned above is allowed to express in the cell.

Further another aspect of the present invention provides a reagent kit, which comprises: the fluorescent protein, the DNA, the recombinant vector, the transformant or the fusion protein according to the present invention as mentioned above.

[Effect of the invention]

[0014]

The present invention provides a novel fluorescent protein which is able to exist in the form of a monomer. When the mitochondria of HeLa cells are labeled with a dimer fluorescent protein KO, the mitochondria are labeled in a granulated state, and thus the original image of such mitochondria cannot be obtained. However, when the mitochondria are labeled with a monomer fluorescent protein mKO, the image of normal narrow filamentous mitochondria is obtained, and the dynamic movement thereof is also observed. Such effectiveness obtained by monomerization was confirmed by the labeling of mitochondrial molecules.

[BEST MODE FOR CARRYING OUT THE INVENTION]

[0015]

The embodiments of the present invention will be described in detail below.

(1) Fluorescent proteins of the present invention

(i) The fluorescent protein of the present invention

The fluorescent protein of the present invention is a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

[0016]

The fluorescent protein of the present invention is characterized in that it has the following properties:

(1) the excitation maximum wavelength is 548 nm, and the fluorescence maximum wavelength is 559 nm;

(2) the molar absorption coefficient at 548 nm is 51,600;

(3) the quantum yield is 0.6; and

(4) the pH sensitivity of fluorescent property is pKa =5.0.

[0017]

Fungia sp. is a certain type of coral. Fungia sp. is characterized in that it lives mainly in the western area of the Atlantic Ocean, in that the contour of a colony thereof is polygonal, in that it has long tentacles, and in that the body as a whole presents bright orange color.

In the examples given below of the present specification, Fungia sp. was used as a starting material, and the fluorescent protein of the present invention having the aforementioned properties was obtained. However, there are cases where the

fluorescent protein of the present invention can also be obtained from coral emitting fluorescence other than Fungia sp. The thus obtained fluorescent protein is also included in the scope of the present invention.

[0018]

The scope of "one or several" in the phrase "an amino acid sequence comprising a deletion, substitution and/or addition of one or several amino acids" used herein is not particularly limited. For example, it means 1 to 20, preferably 1 to 10, more preferably 1 to 7, further preferably 1 to 5, and particularly preferably 1 to 3.

[0019]

The term "equivalent fluorescence properties" is used in the present specification to mean that a fluorescent protein has equivalent fluorescence intensity, equivalent excitation wavelength, equivalent fluorescence wavelength, equivalent pH sensitivity, and the like.

[0020]

The method of obtaining the fluorescent protein of the present invention is not particularly limited. The proteins may be either a protein synthesized by chemosynthesis, or recombinant protein produced by a gene recombination technique.

Where a recombinant protein is produced, it is necessary to obtain DNA encoding the protein. Appropriate primers are designed by using information regarding the amino acid sequence shown in SEQ ID NO 1 of the sequence listing of the present specification and the nucleotide sequence shown in SEQ ID NO 2. Using these primers, PCR is carried out by using cDNA clone of the fluorescent protein described in International Publication WO03/54191 as a template, so that DNA encoding the fluorescent protein of the present invention can be obtained. Where a partial fragment of DNA encoding the fluorescent protein of the present invention is obtained by the above-described PCR, the produced DNA fragments are ligated to one another by a gene recombination technique, so that DNA encoding the desired fluorescent protein can be

obtained. The fluorescent protein of the present invention can be produced by introducing this DNA into an appropriate expression system. Expression in an expression system will be described later in the present specification.

[0021]

(2) DNA of the present invention

The present invention provides genes encoding the fluorescent protein of the present invention.

A specific example of DNA encoding the fluorescent protein of the present invention is DNA encoding a fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

[0022]

A further example of DNA encoding the fluorescent protein of the present invention is DNA described in the following (a) or (b):

(a) DNA having the nucleotide sequence shown in SEQ ID NO: 2; or

(b) DNA, which has a nucleotide sequence comprising a deletion, substitution, and/or addition of one or several nucleotides with respect to the nucleotide sequence shown in SEQ ID NO: 2, and which has a nucleotide sequence encoding a protein that has fluorescence properties equivalent to those of the protein encoded by the nucleotide sequence shown in SEQ ID NO: 2 and that exists in the form of a monomer.

[0023]

The DNA of the present invention can be synthesized by, for example, the phosphoamidite method, or it can also be produced by polymerase chain reaction (PCR)

using specific primers. The DNA of the present invention or its fragment is produced by the method described above in the specification.

[0024]

A method of introducing a desired mutation into a certain nucleic acid sequence is known to a person skilled in the art. For example, known techniques such as a site-directed mutagenesis, PCR using degenerated oligonucleotides, or the exposure of cells containing nucleic acid to mutagens or radioactive rays, are appropriately used, so as to construct DNA having a mutation. Such known techniques are described in, for example, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., 1989; and Current Protocols in Molecular Biology, Supplements 1 to 38, John Wiley & Sons (1987-1997).

[0025]

(3) Recombinant vector of the present invention

The DNA of the present invention can be inserted into a suitable vector and used. The type of a vector used in the present invention is not particularly limited. For example, it may be either a vector that can autonomously replicate (e.g., a plasmid, etc.), or vector that is incorporated into the genomes of host cells when it is introduced into the host cells and is then replicated together with the chromosome into which it is incorporated.

[0026]

The vector used in the present invention is preferably an expression vector. In an expression vector, elements necessary for transcription (e.g., a promoter, etc.) are functionally ligated to the DNA of the present invention. The promoter is a DNA sequence which shows a transcriptional activity in host cells, and it is appropriately selected depending on the type of host cells.

[0027]

Examples of a promoter which can operate in bacterial cells may include a

Bacillus stearothermophilus maltogenic amylase gene promoter, a *Bacillus licheniformis* alpha-amylase gene promoter, a *Bacillus amyloliquefaciens* BAN amylase gene promoter, a *Bacillus subtilis* alkaline protease gene promoter, a *Bacillus pumilus* xylosidase gene promoter, P_R and P_L promoters of phage rhamda, and lac, trp and tac promoters of *Escherichia coli*.

[0028]

Examples of a promoter which can operate in mammalian cells may include an SV40 promoter, an MT-1 (metallothionein gene) promoter, and an adenovirus-2 major late promoter. Examples of a promoter which can operate in insect cells may include a polyhedrin promoter, a P10 promoter, an *Autographa californica* polyhedrosis basic protein promoter, a baculovirus immediate-early gene 1 promoter, and a baculovirus 39K delayed-early gene promoter. Examples of a promoter which can be operate in yeast host cells may include promoters derived from yeast glycolytic genes, an alcohol dehydrogenase gene promoter, a TPI1 promoter, and an ADH2-4c promoter.

Examples of a promoter which can operate in filamentous cells may include an ADH3 promoter and a tpiA promoter.

[0029]

In addition, an appropriate terminator such as a human growth hormone terminator, or a TPI1 terminator or ADH3 terminator for fungal cells, may be functionally bound to the DNA of the present invention, as necessary. The recombinant vector of the present invention may further have elements such as a polyadenylation signal (e.g., one derived from SV40 or the adenovirus 5E1b region), a transcription enhancer sequence (e.g., an SV40 enhancer), or a translation enhancer sequence (e.g., one encoding the adenovirus VA RNA).

The recombinant vector of the present invention may further comprise a DNA sequence which enables the replication of the recombinant vector in host cells. SV40 replication origin is an example of such a sequence (when the host cells are mammalian

cells).

[0030]

The recombinant vector of the present invention may further comprise a selective marker. Examples of such a selective marker may include genes, complements of which are absent from host cells, such as a dihydrofolate reductase (DHFR) gene or a *Shizosaccharomyces pombe* TPI gene, and drug resistant genes such as ampicillin, kanamycin, tetracycline, chloramphenicol, neomycin or hygromycin-resistant genes.

A method for ligating the DNA of the present invention, a promoter and, as desired, a terminator and/or a secretory signal sequence to one another and inserting these items into a suitable vector is known to a person skilled in the art.

[0031]

(4) Transformant of the present invention

A transformant can be produced by introducing the DNA or recombinant vector of the present invention into a suitable host.

Any cell can be used as a host cell into which the DNA or recombinant vector of the present invention is introduced, as long as the DNA construct of the present invention can be expressed therein. Examples of such a cell may include bacteria, yeasts, fungal cells, and higher eukaryotic cells.

[0032]

Examples of bacteria may include Gram-positive bacteria such as Bacillus or Streptomyces, and Gram-negative bacteria such as *Escherichia coli*. These bacteria may be transformed by the protoplast method or other known methods, using competent cells.

Examples of mammalian cells may include HEK 293 cells, HeLa cells, COS cells, BHK cells, CHL cells, and CHO cells. A method of transforming mammalian cells and expressing the introduced DNA sequence in the cells is also known. Examples

of such a method may include the electroporation, the calcium phosphate method, and the lipofection method.

[0033]

Examples of yeast cells may include those belonging to Saccharomyces or Shizosaccharomyces. Examples of such cells may include *Saccharomyces cerevisiae* and *Saccharomyces kluyveri*. Examples of a method of introducing a recombinant vector into yeast host cells may include the electroporation, the spheroplast method, and the lithium acetate method.

[0034]

Examples of other fungal cells may include those belonging to *Filamentous fungi* such as Aspergillus, Neurospora, Fusarium or Trichoderma. Where *Filamentous fungi* are used as host cells, transformation can be carried out by incorporating DNA constructs into host chromosomes, so as to obtain recombinant host cells. Incorporation of DNA constructs into the host chromosomes is carried out by known methods, and such known methods may include homologous recombination and heterologous recombination.

[0035]

Where insect cells are used as host cells, both a vector into which a recombinant gene is introduced and a baculovirus are co-introduced into insect cells, and a recombinant virus is obtained in the culture supernatant of the insect cells. Thereafter, insect cells are infected with the recombinant virus, so as to allow the cells to express proteins (described in, for example, Baculovirus Expression Vectors, A Laboratory Manual; and Current Protocols in Molecular Biology, Bio/Technology, 6, 47 (1988)).

[0036]

The *Autographa californica* nuclear polyhedrosis virus, which is a virus infecting to insects belonging to *Barathra brassicae*, can be used as baculovirus.

Examples of insect cells used herein may include Sf9 and Sf21, which are

Spodoptera frugiperda ovarian cells [Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman & Company, New York, (1992)], and HiFive (manufactured by Invitrogen), which are *Trichoplusia ni* ovarian cells.

Examples of the method of co-introducing both a vector into which a recombinant gene has been introduced and the above baculovirus into insect cells to prepare a recombinant virus may include the calcium phosphate method and the lipofection method.

[0037]

The above transformant is cultured in an appropriate nutritive medium under conditions enabling the introduced DNA construct to be expressed. In order to isolate and purify the protein of the present invention from the culture product of the transformant, common methods of isolating and purifying proteins may be used.

For example, where the protein of the present invention is expressed in a state dissolved in cells, after completion of the culture, cells are recovered by centrifugal separation, and the recovered cells are suspended in a water type buffer. Thereafter, the cells are disintegrated using an ultrasonic disintegrator or the like, so as to obtain a cell-free extract. A supernatant is obtained by centrifuging the cell-free extract, and then, a purified sample can be obtained from the supernatant by applying, singly or in combination, the following ordinary protein isolation and purification methods: the solvent extraction, the salting-out method using ammonium sulfate or the like, the desalting method, the precipitation method using an organic solvent, the anion exchange chromatography using resins such as diethylaminoethyl (DEAE) sepharose, the cation exchange chromatography using resins such as S-Sepharose FF (manufactured by Pharmacia), the hydrophobic chromatography using resins such as butyl sepharose or phenyl sepharose, the gel filtration method using a molecular sieve, the affinity chromatography, the chromatofocusing method, and the electrophoresis such as isoelectric focusing.

[0038]

(5) Use of the fluorescent protein of the present invention and a fusion fluorescent protein comprising the same

The fluorescent protein of the present invention can be fused with another protein, so as to construct a fusion fluorescent protein.

A method of obtaining the fusion fluorescent protein of the present invention is not particularly limited. It may be either a protein synthesized by chemosynthesis, or recombinant protein produced by a gene recombination technique.

Where a recombinant protein is produced, it is necessary to obtain DNA encoding the protein. Appropriate primers are designed using the information regarding the amino acid sequence shown in SEQ ID NO: 1 of the sequence listing of the present specification and the nucleotide sequence shown in SEQ ID NO: 2. Using these primers, PCR is carried out using a DNA fragment containing the gene of the fluorescent protein of the present invention as a template, so as to produce DNA fragments necessary for construction of the DNA encoding the fluorescent protein of the present invention. Moreover, DNA fragment encoding a protein to be fused is also obtained in the same above manner.

[0039]

Subsequently, the thus obtained DNA fragments are ligated to one another by a gene recombination technique, so that DNA encoding the desired fusion fluorescent protein can be obtained. This DNA is then introduced into an appropriate expression system, so that the fusion fluorescent protein of the present invention can be produced.

[0040]

The fluorescent protein of the present invention has an extremely high utility value as a marker. This is to say, the fluorescent protein of the present invention is purified as a fusion protein with an amino acid sequence to be tested, and the fusion protein is introduced into cells by methods such as the microinjection. By observing

the distribution of the fusion protein over time, targeting activity of the amino acid sequence to be tested can be detected in the cells.

[0041]

The type of another protein (an amino acid sequence to be tested) with which the fluorescent protein of the present invention is fused is not particularly limited. Preferred examples may include proteins localizing in cells, proteins specific for intracellular organelles, and targeting signals (e.g., a nuclear transport signal, a mitochondrial presequence, etc.). In addition, the fluorescent protein of the present invention can be expressed in cells and used, as well as being introduced into cells by the microinjection or the like. In this case, a vector into which the DNA encoding the fluorescent protein of the present invention is inserted in such a way that it can be expressed, is introduced into host cells.

[0042]

Moreover, the fluorescent protein of the present invention can also be used as a reporter protein to determine promoter activity. This is to say, a vector is constructed such that DNA encoding the fluorescent protein of the present invention is located downstream of a promoter to be tested, and the vector is then introduced into host cells. By detecting the fluorescence of the fluorescent protein of the present invention which is emitted from the cells, the activity of the promoter to be tested can be determined. The type of a promoter to be tested is not particularly limited, as long as it operates in host cells.

[0043]

A vector used to detect the targeting activity of the above amino acid sequence to be tested or to determine promoter activity is not particularly limited. Examples of a vector preferably used for animal cells may include pNEO (P. Southern, and P. Berg (1982) J. Mol. Appl. Genet. 1: 327), pCAGGS (H. Niwa, K. Yamamura, and J. Miyazaki, Gene 108, 193-200 (1991)), pRc/CMV (manufactured by Invitrogen), and pCDM8

(manufactured by Invitrogen). Examples of a vector preferably used for yeasts may include pRS303, pRS304, pRS305, pRS306, pRS313, pRS314, pRS315, pRS316 (R. S. Sikorski and P. Hieter (1989) Genetics 122: 19-27), pRS423, pRS424, pRS425, pRS426 (T. W. Christianson, R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter (1992) Gene 110: 119-122).

[0044]

In addition, the type of cells used herein is also not particularly limited. Various types of animal cells such as L cells, BalbC-3T3 cells, NIH3T3 cells, CHO (Chinese hamster ovary) cells, HeLa cells or NRK (normal rat kidney) cells, yeast cells such as *Saccharomyces cerevisiae*, *Escherichia coli* cells, or the like can be used. Vector can be introduced into host cells by common methods such as the calcium phosphate method or the electroporation.

[0045]

The above obtained fusion fluorescent protein of the present invention wherein the fluorescent protein of the present invention is fused with another protein (referred to as a protein X) is allowed to be expressed in cells. By monitoring a fluorescence emitted, it becomes possible to analyze the localization or dynamics of the protein X in cells. That is, cells transformed or transfected with DNA encoding the fusion fluorescent protein of the present invention are observed with a fluorescence microscope, so that the localization and dynamics of the protein X in the cells can be visualized and thus analyzed.

[0046]

For example, by using a protein specific for an intracellular organella as a protein X, the distribution and movement of a nucleus, a mitochondria, an endoplasmic reticulum, a Golgi body, a secretory vesicle, a peroxisome, etc., can be observed.

Moreover, for example, axis cylinders or dendrites of the nerve cells show an extremely complicated change in strikes in an individual who is under development.

Accordingly, fluorescent labeling of these sites enables a dynamic analysis.

[0047]

The fluorescence of the fluorescent protein of the present invention can be detected with a viable cell. Such detection can be carried out using, for example, a fluorescence microscope (Axiophoto Filter Set 09 manufactured by Carl Zeiss) or an image analyzer (Digital Image Analyzer manufactured by ATTO).

The type of a microscope can be appropriately selected depending on purposes. Where frequent observation such as pursuit of a change over time is carried out, an ordinary incident-light fluorescence microscope is preferable. Where observation is carried out while resolution is emphasized, for example, in the case of searching localization in cells specifically, a confocal laser scanning microscope is preferable. In terms of maintenance of the physiological state of cells and prevention from contamination, an inverted microscope is preferable as a microscope system. When an erecting microscope with a high-powered lens is used, a water immersion lens can be used.

[0048]

A filter set can be appropriately selected depending on the fluorescence wavelength of a fluorescent protein. Since the fluorescent protein of the present invention has an excitation maximum wavelength of 548 nm, and a fluorescence maximum wavelength of 559 nm, a filter having an excitation light between approximately 530 and 550 nm and a fluorescence between approximately 550 and 600 nm is preferably used.

[0049]

When viable cells are observed over time using a fluorescence microscope, a high sensitive cooled CCD camera is used, since photography is carried out in a short time. In the case of the cooled CCD camera, CCD is cooled to decrease thermal noise, so that a weak fluorescence image can be clearly photographed by exposure in a short

time.

[0050]

(6) Kit of the present invention

The present invention provides a kit for analyzing the localization of intracellular components and/or analyzing physiologically active substances, which is characterized in that it comprises at least one selected from the fluorescent protein, the fusion fluorescent protein, the DNA, the recombinant vector, or the transformant, which are described in the present specification. The kit of the present invention can be produced from commonly used materials that are known per se, by using common methods.

Reagents such as the fluorescent protein or the DNA are dissolved in an appropriate solvent, so that the reagents can be prepared in a form suitable for conservation. Water, ethanol, various types of buffer solution, etc. can be used as such a solvent.

The present invention will be further described in the following examples. However, the present invention is not limited by these examples.

[EXAMPLES]

[0051]

Example 1: Production of multimer formation-inhibiting mutant by point mutation introduction

A multimer interface was predicted from the amino acid sequence of KO-1, and the amino acids of the multimer interface were substituted with other amino acids. Moreover, KO-1 was monomerized, so that it could maintain fluorescence properties. Point mutation introduction was carried out, using an *Escherichia coli* expression vector (pRSET $_B$) (an expression vector having DNA encoding KO-1 described in International Publication WO03/54191), into which KO-1 had been inserted, and also using point mutation introduction primers. Specifically, multiple mutation introduction primers

were simultaneously annealed on one side chain of a template plasmid, followed by elongation with polymerase. DNA fragments elongated with each primers were ligated to one another using DNA ligase in the same reaction solution, so as to obtain a product, whose portions other than the mutation-introduced portion were complementary to the template. Since the termini of the DNA fragments needed phosphate groups when the fragments were ligated to one another with DNA ligase, the 5'-terminal sides of the used primers had been subjected to phosphorylation.

(1) 5'-phosphorylation of primers

100 μM primers	2 μl
10 x T4 polynucleotide kinase buffer	5 μl
100 μM ATP	0.5 μl
Sterilized water	41.5 μl
T4 polynucleotide kinase (10 U/μl)	1 μl

[0052]

The above mixture was incubated at 37°C for 30 minutes. As primers used herein, the following primers having the nucleotide sequences shown in SEQ ID NOS: 3 to 17 were used.

K11R, F13Y

CCAGAGATGAAGATGAGGTACTACATGGACGGC (SEQ ID NO:3)

V25I

CATGAGTTCACAATTGAAGGTGAAGGC (SEQ ID NO:4)

K32R

GAAGGCACAGGCAGACCTTACGAGGGA (SEQ ID NO:5)

S55A

CCAATGCCTTTCGCGTTTGACTTAGTG (SEQ ID NO:6)

T62V

TTAGTGTCACACGTGTTCTGTTACGGC (SEQ ID NO:7)

Q96E

GAAAGGTCGTTGGAGTTCGAAGATGGT（SEQ ID NO:8）

F102S, A104S

GAAGATGGTGGGTCCGCTTCAGTCAGTGCG（SEQ ID NO:9）

C115T, E117Y

AGCCTTAGAGGAAACACCTTCTACCACAAATCCA（SEQ ID NO:10）

V123T

CAAATCCAAATTTACTGGGGTTAACTTTCCTG（SEQ ID NO:11）

V133I

GCCGATGGTCCTATCATGCAAAACCAAAGT（SEQ ID NO:12）

S139V

GCCGATGGTCCTATCATGCAAAACCAAAGTGTTGATTGGGAGCCA(SEQ ID NO:13）

T150A, C151S

GAGAAAATTACTGCCAGCGACGGAGTTCTGAAG（SEQ ID NO:14）

F162Y, A166E

GATGTTACGATGTACCTAAAACTTGAAGGAGGCGGCAATCAC（SEQ ID NO:15）

Q190G, F193Y, G195S

CTTAAAATGCCAGGAAGCCATTACATCAGCCATCGCCTCGTCAGG（SEQ ID NO:16）

C217S

GATGCAGTAGCTCATTCCCTCGAGCACCACCACC（SEQ ID NO:17）

[0053]

(2) Point mutation introduction PCR

5'-phosphorylated primers	4 µl
Template (KO-pRSET $_B$)	100 ng
10 x polymerase buffer	2.5 µl

10 x DNA ligase buffer	2.5 μl
2.5 mM dNTPs	1 μl
polymerase (pfu) 2.5U/μl	1 μl
Taq DNA ligase 40U/μl	0.5 μl

The final volume of the mixture was set at 50 μl by addition of sterilized water.

[0054]

Program:

GeneAmp PCR system 9700 was used as a thermal cycler.

(1) 65°C	5 min
(2) 95°C	2 min
(3) 95°C	20 sec
(4) 52°C	20 sec
(5) 65°C	8 min

The operation described in (3) to (5) above was repeated for 25 cycles.

(6) 75°C	7 min
(7) 4°C	hold

[0055]

(3) Dpn1 treatment

1 μl of Dpn1 was added to the sample obtained after PCR, and the obtained mixture was then incubated at 37°C for 1 hour, so as to cleave a template plasmid.

[0056]

(4) Transformation of *Escherichia coli*

Escherichia coli JM109 was transformed with the sample treated with Dpn1, so that KO-1 after introduction of the mutation was allowed to express therein.

[0057]

(5) Amino acid sequence of monomerized Kusabira-Orange (mKO)

The nucleotide sequence of the KO mutant obtained after introduction of the

mutation was analyzed, so as to determine the amino acid sequence thereof. As a result, it was found that lysine (K) at position 11 was substituted with arginine (R), phenylalanine (F) at position 13 was substituted with tyrosine (Y), valine (V) at position 25 was substituted with isoleucine (I), lysine (K) at position 32 was substituted with arginine (R), serine (S) at position 55 was substituted with alanine (A), threonine (T) at position 62 was substituted with valine (V), glutamine (Q) at position 96 was substituted with glutamic acid (E), phenylalanine (F) at position 102 was substituted with serine (S), alanine (A) at position 104 was substituted with serine (S), cysteine (C) at position 115 was substituted with threonine (T), glutamic acid (E) at position 117 was substituted with tyrosine (Y), valine (V) at position 123 was substituted with threonine (T), valine (V) at position 133 was substituted with isoleucine (I), serine (S) at position 139 was substituted with valine (V), threonine (T) at position 150 was substituted with alanine (A), cysteine (C) at position 151 was substituted with serine (S), phenylalanine (F) at position 162 was substituted with tyrosine (Y), alanine (A) at position 166 was substituted with glutamic acid (E), glutamine (Q) at position 190 was substituted with glycine (G), phenylalanine (F) at position 193 was substituted with tyrosine (Y), glycine (G) at position 195 was substituted with serine (S), and cysteine (C) at position 217 was substituted with serine (S). Moreover, in order to add the Kozak sequence, valine (V) was introduced into the position before serine (S) at position 2. This mutant was named as mKO. The amino acid sequence of mKO is shown in SEQ ID NO: 1 of the sequence listing, and the nucleotide sequence thereof is shown in SEQ ID NO: 2 of the sequence listing.

[0058]

A protein formed by adding His-Tag to mKO was allowed to express in *Escherichia coli* according to common methods, and it was then purified with Ni-Agarose.

[0059]

Example 2: Analysis of fluorescence properties

The fluorescence and absorption spectra of the mKO protein purified in Example 1 were measured as follows. The quantum yield and molar absorption coefficient thereof were calculated.

An absorption spectrum was measured using a 20 μM fluorescent protein and a 50 mM HEPES solution (pH 7.5). A molar absorption coefficient was calculated from the peak value of this spectrum. In the case of mKO, the fluorescent protein was diluted with the aforementioned buffer solution such that the absorption peak was found at 548 nm and such that absorption at 500 nm became 0.0025. Thereafter, the fluorescence spectrum obtained by excitation at 500 nm, and the excitation spectrum obtained by fluorescence at 590 nm, were measured. The fluorescence spectrum of DsRed (CLONTECH) was also measured under conditions wherein the absorption at 500 nm became 0.0025. The quantum yield of DsRed was set at 0.29, and the quantum yield of mKO was obtained.

The results are shown in Table 1, and Figures 1 and 2. Table 1 also shows the data of the KO protein (dimer protein) described in International Publication WO03/54191.

[0060]

[Table 1]

Table 1

	Maximum excitation	Maximum fluorescence	Molar absorption coefficient	Quantum yield	Number of amino acids	multimer formation	pH sensitivity
KO	548 nm	561 nm	109750	0.45	217	Dimer	pKa<5.0
mKO	548 nm	559 nm	51600	0.6	218	Monomer	PKa=5.0

[0061]

Example 3: Measurement of molecular weight by ultracentrifugal analysis

An mKO protein solution with the composition consisting of 150 mM KCl and 50 mM HEPES-KOH (pH 7.4) was prepared. The molecular weight of mKO was determined by ultracentrifugal analysis. The above solution was centrifuged with an ultracentrifuge XL-1 (Beckman Coulter) at 25,000 rpm for 22 hours, so as to measure absorption at 540 nm around the maximum absorption (548 nm) of mKO. From the measurement results, the molecular weight of mKO was calculated to be 28 kDa (Figure 3). This value was almost the same as 26 kDa predicted from the amino acid sequence, and thus it was confirmed that mKO exists in the form of a monomer.

[0062]

Example 4: Targeting to mitochondria

12 amino acids (MLSLRQSIRFFK) at the N-terminus of cytochrome oxidase subunit 4 derived from yeast were added to each of the N-termini of KO and mKO. Thereafter, targeting to the mitochondria of HeLa cells was conducted, so as to label the mitochondria. As a result, it was confirmed that KO (dimer) was not exactly targeted to the mitochondria, and that the mitochondria was stained in a granulated state (Figure 4). On the other hand, mKO (monomer) was exactly targeted to the mitochondria, and narrow filamentous mitochondria were observed. Thus, effectiveness obtained by monomerization was confirmed (Figure 5).

[BRIEF DESCRIPTION OF THE DRAWINGS]

[0063]

[Figure 1] Figure 1 shows the absorption spectrum of mKO.

[Figure 2] Figure 2 shows the excitation spectrum (dotted line) and fluorescence spectrum (solid line) of mKO.

[Figure 3] Figure 3 shows the results of molecular weight measurement by ultracentrifugation. From the measurement results, the molecular weight was found to be 28 kDa.

[Figure 4] Figure 4 shows the results obtained by labeling the mitochondria of HeLa cells

with KO (dimer). The mitochondria were converted to granules, which differ from the form of normal mitochondria.

[Figure 5] Figure 5 shows the results obtained by labeling the mitochondria of HeLa cells with mKO (monomer). The mitochondria had a normal corded form.

[SEQUENCE LISTING]

[0064]

SEQUENCE LISTING

<110> RIKEN

<120> Fluorescent protein

<130> A31701A

<160> 17

<210> 1

<211> 218

<212> PRT

<213> Fungia sp.

<400> 1

Met Val Ser Val Ile Lys Pro Glu Met Lys Met Arg Tyr Tyr Met Asp
1 5 10 15

Gly Ser Val Asn Gly His Glu Phe Thr Ile Glu Gly Glu Gly Thr Gly
20 25 30

Arg Pro Tyr Glu Gly His Gln Glu Met Thr Leu Arg Val Thr Met Ala
35 40 45

Lys Gly Gly Pro Met Pro Phe Ala Phe Asp Leu Val Ser His Val Phe
50 55 60

Cys Tyr Gly His Arg Pro Phe Thr Lys Tyr Pro Glu Glu Ile Pro Asp
65 70 75 80

Tyr Phe Lys Gln Ala Phe Pro Glu Gly Leu Ser Trp Glu Arg Ser Leu
85 90 95

Glu Phe Glu Asp Gly Gly Ser Ala Ser Val Ser Ala His Ile Ser Leu
100 105 110

Arg Gly Asn Thr Phe Tyr His Lys Ser Lys Phe Thr Gly Val Asn Phe

115 120 125

Pro Ala Asp Gly Pro Ile Met Gln Asn Gln Ser Val Asp Trp Glu Pro

130 135 140

Ser Thr Glu Lys Ile Thr Ala Ser Asp Gly Val Leu Lys Gly Asp Val

145 150 155 160

Thr Met Tyr Leu Lys Leu Glu Gly Gly Gly Asn His Lys Cys Gln Phe

165 170 175

Lys Thr Thr Tyr Lys Ala Ala Lys Lys Ile Leu Lys Met Pro Gly Ser

180 185 190

His Tyr Ile Ser His Arg Leu Val Arg Lys Thr Glu Gly Asn Ile Thr

195 200 205

Glu Leu Val Glu Asp Ala Val Ala His Ser

210 215

<210> 2

<211> 657

<212> DNA

<213> Fungia sp.

<400> 2

atg gtg agt gtg att aaa cca gag atg aag atg agg tac tac atg gac 48
Met Val Ser Val Ile Lys Pro Glu Met Lys Met Arg Tyr Tyr Met Asp

1 5 10 15

ggc tcc gtc aat ggg cat gag ttc aca att gaa ggt gaa ggc aca ggc 96
Gly Ser Val Asn Gly His Glu Phe Thr Ile Glu Gly Glu Gly Thr Gly

20 25 30

aga cct tac gag gga cat caa gag atg aca cta cgc gtc aca atg gcc 144
Arg Pro Tyr Glu Gly His Gln Glu Met Thr Leu Arg Val Thr Met Ala

35 40 45

aag ggc ggg cca atg cct ttc gcg ttt gac tta gtg tca cac gtg ttc 192
Lys Gly Gly Pro Met Pro Phe Ala Phe Asp Leu Val Ser His Val Phe

50 55 60

tgt tac ggc cac aga cct ttt act aaa tat cca gaa gag ata cca gac 240
Cys Tyr Gly His Arg Pro Phe Thr Lys Tyr Pro Glu Glu Ile Pro Asp

65 70 75 80

tat ttc aaa caa gca ttt cct gaa ggc ctg tca tgg gaa agg tcg ttg 288
Tyr Phe Lys Gln Ala Phe Pro Glu Gly Leu Ser Trp Glu Arg Ser Leu

85 90 95

gag ttc gaa gat ggt ggg tcc gct tca gtc agt gcg cat ata agc ctt 336
Glu Phe Glu Asp Gly Gly Ser Ala Ser Val Ser Ala His Ile Ser Leu

100 105 110

aga gga aac acc ttc tac cac aaa tcc aaa ttt act ggg gtt aac ttt 384
Arg Gly Asn Thr Phe Tyr His Lys Ser Lys Phe Thr Gly Val Asn Phe

115 120 125

cct gcc gat ggt cct atc atg caa aac caa agt gtt gat tgg gag cca 432
Pro Ala Asp Gly Pro Ile Met Gln Asn Gln Ser Val Asp Trp Glu Pro

130 135 140

tca acc gag aaa att act gcc agc gac gga gtt ctg aag ggt gat gtt 480
Ser Thr Glu Lys Ile Thr Ala Ser Asp Gly Val Leu Lys Gly Asp Val

145 150 155 160

acg atg tac cta aaa ctt gaa gga ggc ggc aat cac aaa tgc caa ttc 528
Thr Met Tyr Leu Lys Leu Glu Gly Gly Gly Asn His Lys Cys Gln Phe

165 170 175

aag act act tac aag gcg gca aaa aag att ctt aaa atg cca gga agc 576
Lys Thr Thr Tyr Lys Ala Ala Lys Lys Ile Leu Lys Met Pro Gly Ser

180 185 190

cat tac atc agc cat cgc ctc gtc agg aaa acc gaa ggc aac att act 624
His Tyr Ile Ser His Arg Leu Val Arg Lys Thr Glu Gly Asn Ile Thr
195 200 205

gag ctg gta gaa gat gca gta gct cat tcc tga 657
Glu Leu Val Glu Asp Ala Val Ala His Ser
210 215

<210> 3

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 3

ccagagatga agatgaggta ctacatggac ggc 33

<210> 4

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 4

catgagttca caattgaagg tgaaggc 27

<210> 5

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 5

gaaggcacag gcagacctta cgaggga 27

<210> 6

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 6

ccaatgcctt tcgcgtttga cttagtg 27

<210> 7

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 7

ttagtgtcac acgtgttctg ttacggc 27

<210> 8

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 8

gaaaggtcgt tggagttcga agatggt 27

<210> 9

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 9

gaagatggtg ggtccgcttc agtcagtgcg 30

<210> 10

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 10

agccttagag gaaacacctt ctaccacaaa tcca 34

<210> 11

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 11

caaatccaaa tttactgggg ttaactttcc tg 32

<210> 12

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 12

gccgatggtc ctatcatgca aaaccaaagt 30

<210> 13

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 13

gccgatggtc ctatcatgca aaaccaaagt gttgattggg agcca 45

<210> 14

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 14

gagaaaatta ctgccagcga cggagttctg aag 33

<210> 15

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 15

gatgttacga tgtacctaaa acttgaagga ggcggcaatc ac 42

<210> 16

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 16

cttaaaatgc caggaagcca ttacatcagc catcgcctcg tcagg 45

<210> 17

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 17

gatgcagtag ctcattccct cgagcaccac cacc 34

[Name of Document] Drawings

[Fig.1]

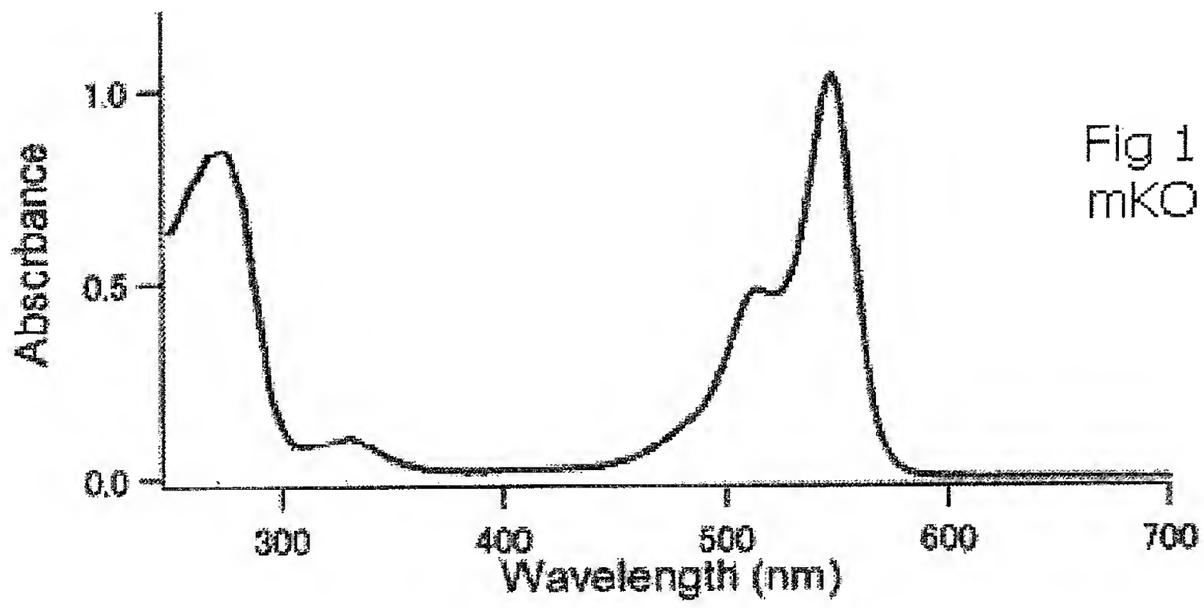


Fig 1
mKO absorption spectrum

[Fig.2]

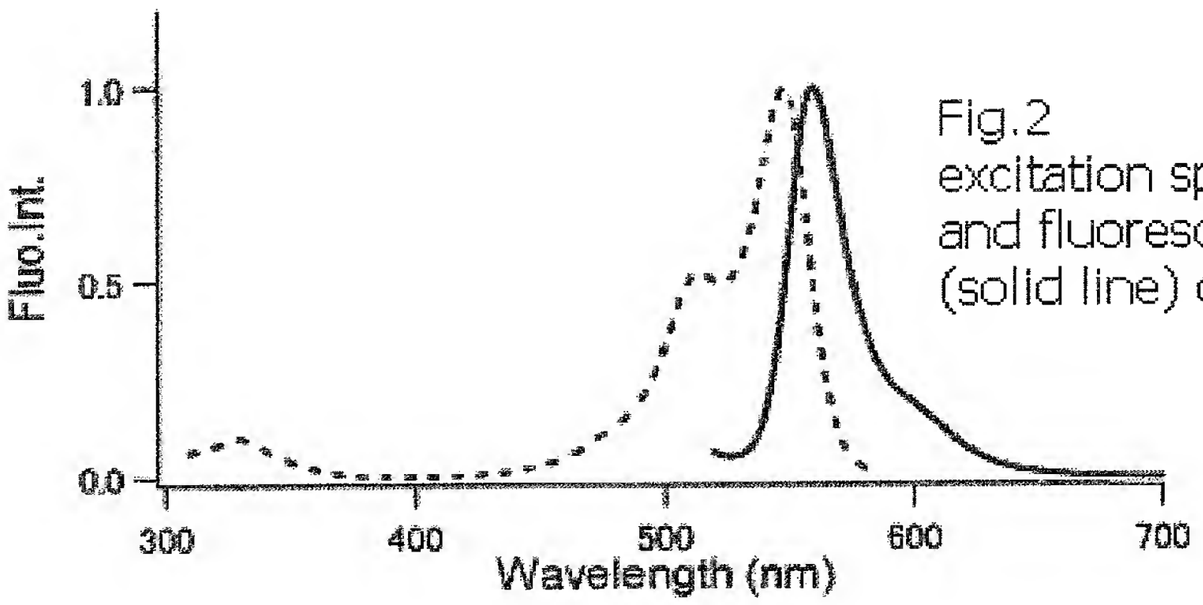
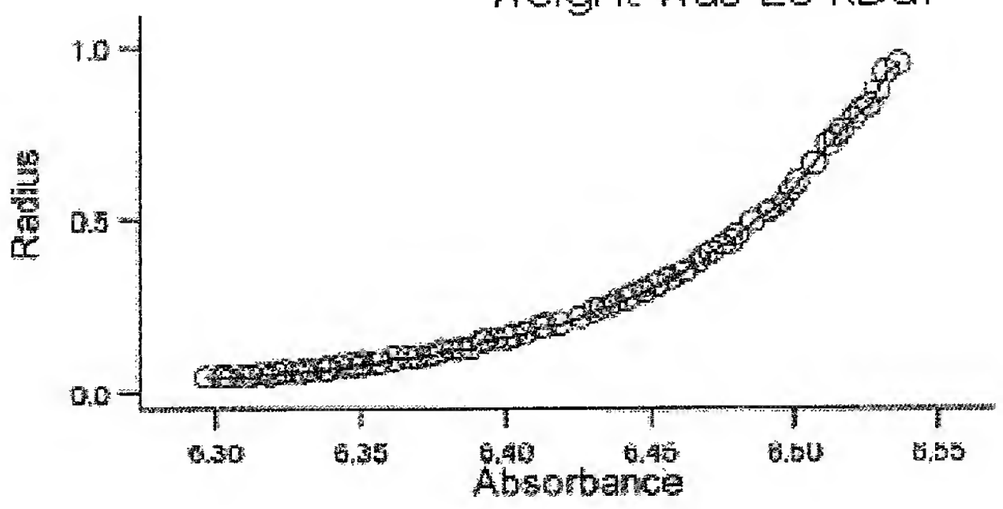


Fig.2
excitation spectrum (dotted line)
and fluorescence spectrum
(solid line) of mKO

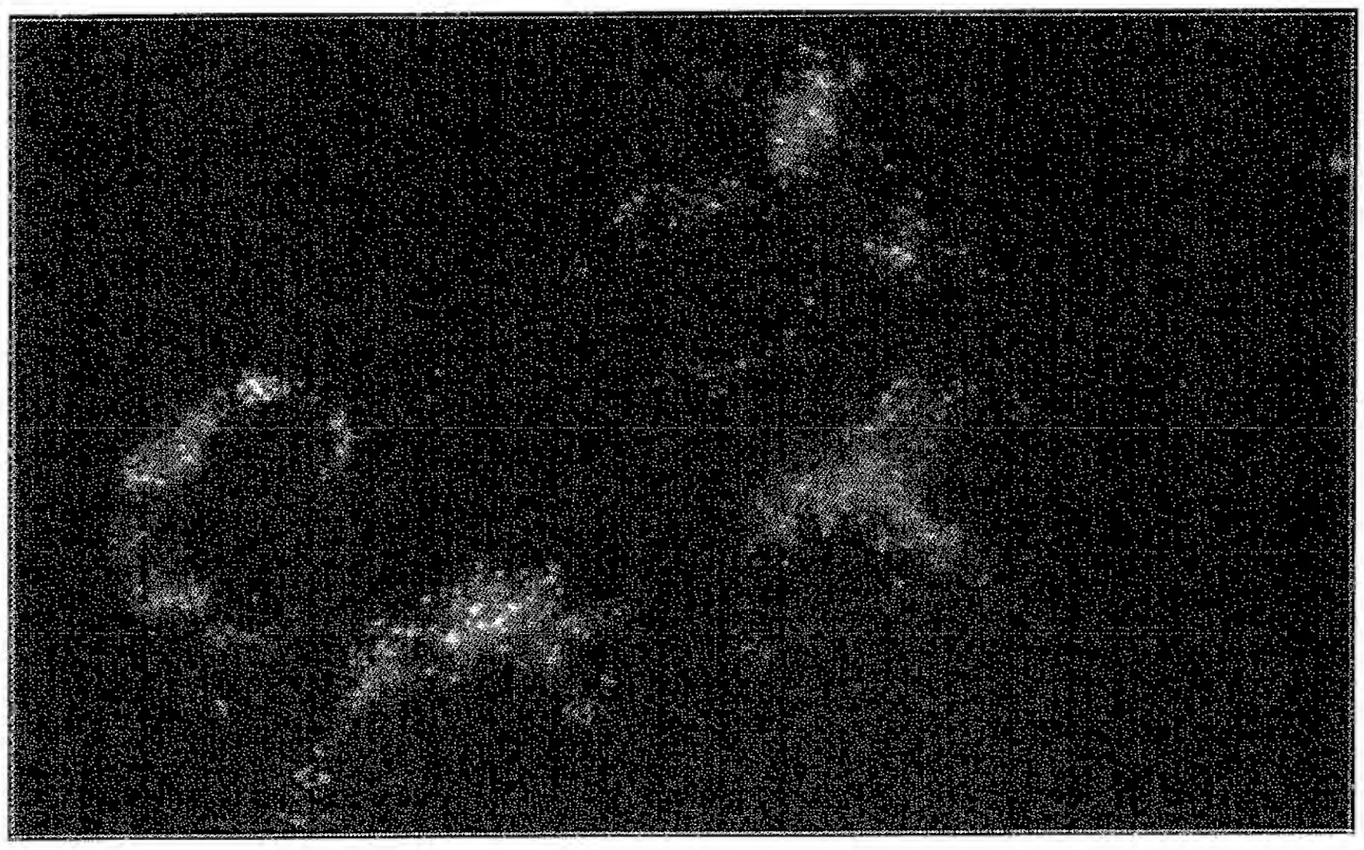
[Fig.3]

Fig.3 Molecular weight measurement by ultracentrifugation
From measurement results, the molecular weight was 28 kDa.



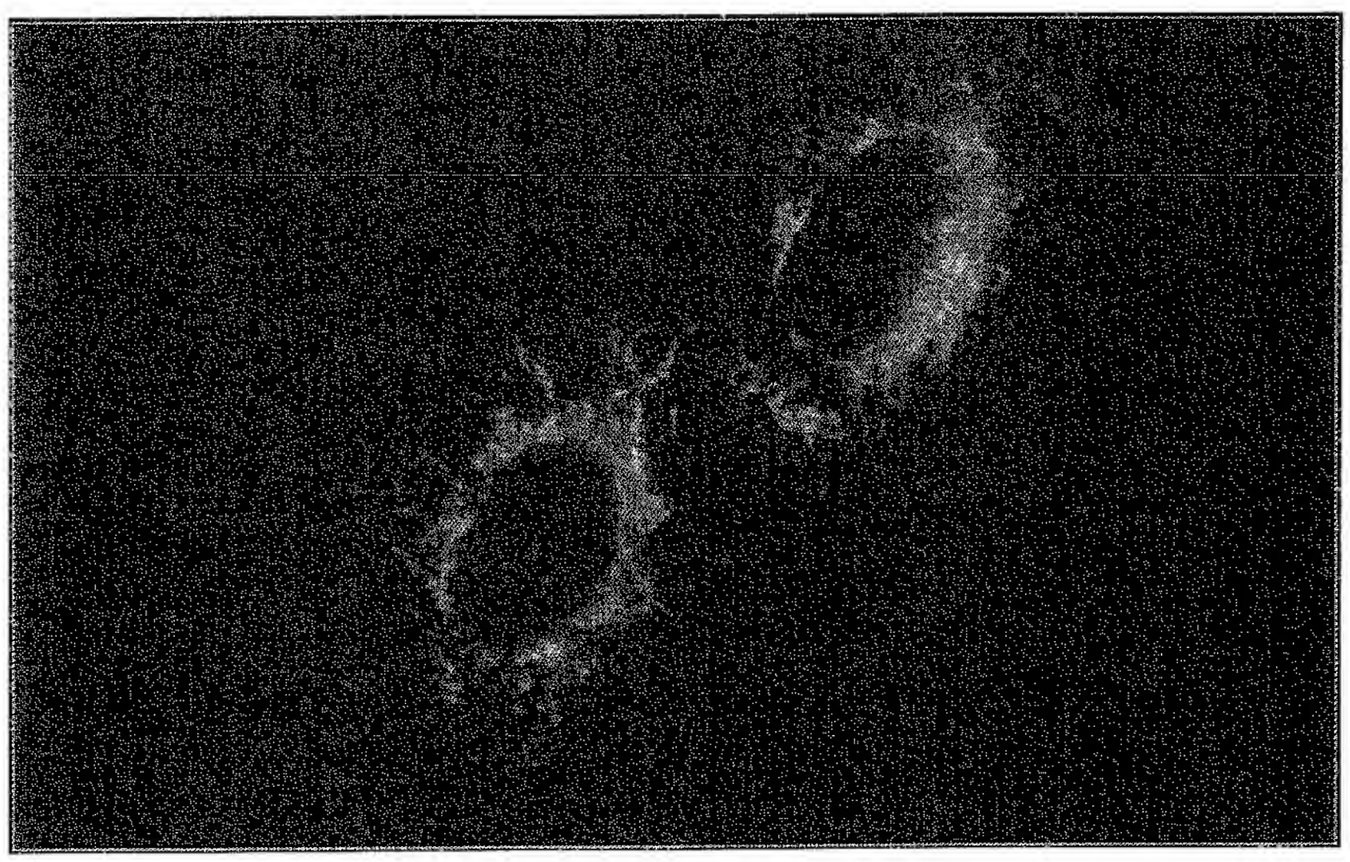
[Fig.4]

Figure 4
Labeling of mitochondria of HeLa cells with KO (dimer)
The mitochondria were converted to granules, which differ from the form of normal mitochondria.



[Fig.5]

Figure 5
Labeling of the mitochondria of HeLa cells with mKO (monomer).
The mitochondria was observed to be a normal corded form.



[Name of Document] ABSTRACT

[Abstract]

[Object] To provide a novel fluorescent protein, which exists in the form of a monomer without forming a multimer.

[Means for solution] A fluorescent protein described in the following (a) or (b):

(a) a protein having the amino acid sequence shown in SEQ ID NO: 1; or

(b) a protein, which has an amino acid sequence comprising a deletion, substitution, and/or addition of one or several amino acids with respect to the amino acid sequence shown in SEQ ID NO: 1, which has fluorescence properties equivalent to those of the protein having the amino acid sequence shown in SEQ ID NO: 1, and which exists in the form of a monomer.

[Selected Drawing] None